

brain and other tissues: humoral factors and neuronal pathways. Leptin, a humoral factor from adipocytes, is a mediator of metabolic information from adipose tissue to the hypothalamus (2). In addition, circulating nutrients reportedly affect food intake and alter hepatic glucose production via the efferent vagal pathway (23, 24). An afferent vagal signal originating in the liver is likely to be another metabolic information pathway. In this way, the brain may integrate information obtained from several tissues and organs via both humoral and neuronal pathways. When the brain receives information regarding excess energy storage, the sympathetic nervous system is activated to enhance energy expenditure and lipolysis, thereby maintaining energy homeostasis. Disturbance of the control system is implicated in the development of the metabolic syndrome (25). Targeting of this neuronal pathway is a potential therapeutic strategy for treating the metabolic syndrome.

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## Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S6

Table S1

References

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# Synaptic Amplifier of Inflammatory Pain in the Spinal Dorsal Horn

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Inflammation and trauma lead to enhanced pain sensitivity (hyperalgesia), which is in part due to altered sensory processing in the spinal cord. The synaptic hypothesis of hyperalgesia, which postulates that hyperalgesia is induced by the activity-dependent long-term potentiation (LTP) in the spinal cord, has been challenged, because in previous studies of pain pathways, LTP was experimentally induced by nerve stimulation at high frequencies (~100 hertz). This does not, however, resemble the real low-frequency afferent barrage that occurs during inflammation. We identified a synaptic amplifier at the origin of an ascending pain pathway that is switched-on by low-level activity in nociceptive nerve fibers. This model integrates known signal transduction pathways of hyperalgesia without contradiction.

Inflammation of peripheral tissues causes spontaneous pain and hyperalgesia. Amplification of pain-related information in the spinal dorsal horn lamina I contributes to inflammatory pain (1–6). Inflammation causes release of neuromodulators, including substance P and glutamate in spinal dorsal horn (7, 8), potentially leading to  $\text{Ca}^{2+}$ -dependent LTP. In all previous studies, spinal LTP was induced by brief (1 s), high-frequency (100 Hz) burstlike stimulation (HFS) of afferent nerve fibers. High-frequency bursts do not, however, resemble the continuous low-frequency afferent barrage that occurs during inflammation. Low-frequency presynaptic activity normally fails to

induce LTP but rather induces synaptic long-term depression (LTD) (9). The LTP model of inflammatory hyperalgesia thus may be questioned. Here, we evaluated the effect of low-frequency afferent barrage on synaptic transmission in ascending pain pathways and asked if synaptic plasticity is differentially induced in distinct ascending pain tracts. We labeled lamina I projection neurons by retrograde fluorescent marker DiI (1,1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), injected into either of two major projection areas of spinal lamina I neurons: the parabrachial (PB) area or the periaqueductal gray (PAG) (10, 11) (Fig. 1, A and B). To circumvent confounding developmental factors, we used only juvenile or adult rats in this study. Transverse spinal cord slices with long dorsal roots attached were prepared 3 to 4 days after DiI injections to allow whole-cell recordings from identified projection neurons in 21- to 28-day-old rats (10). In the presence of tetrodotoxin, bath application of substance P

(2  $\mu\text{M}$ ) induced transient inward currents in 21 out of 27 spino-PB and in 9 out of 12 spino-PAG neurons (Fig. 1C), confirming the expression of functional neurokinin 1 receptors (NK1Rs). Spinal release of substance P following electrical stimulation of primary afferents at C-fiber strength was assessed by the internalization of NK1R in lamina I neurons. HFS parameters (100-Hz bursts) similar to all previously used conditioning stimulation protocols to induce classical LTP in pain pathways, or low-frequency stimulation (LFS, 2 Hz), was used. Both types of stimulation elicited substantial NK1R internalization in  $89 \pm 1\%$  and in  $78 \pm 4\%$  of 150 neurons evaluated in three rats per group (Fig. 1D). We then used these stimulation protocols for conditioning.

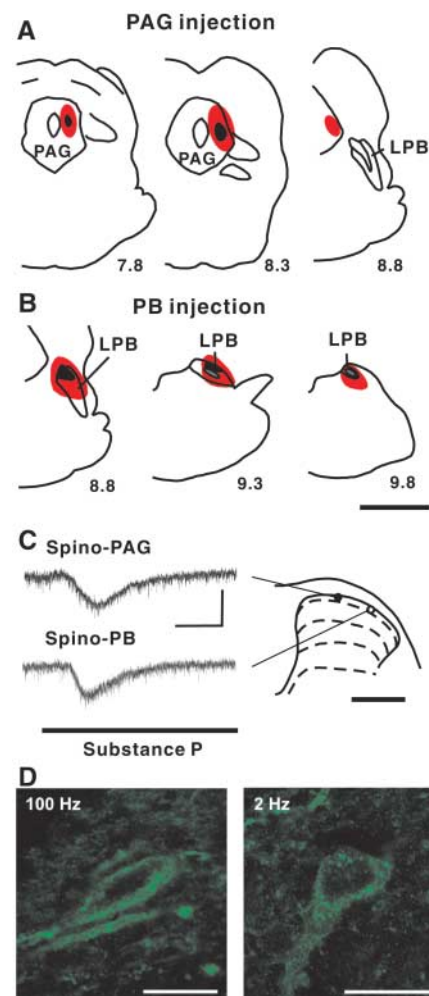
Conditioning HFS induces LTP at synapses between C-fibers and lamina I neurons that project to the PB (12). We confirmed these results by showing LTP of monosynaptically evoked excitatory postsynaptic currents (EPSCs) to  $172 \pm 15\%$  of the control value at 30 min after conditioning ( $n = 8$ ) (Fig. 2A). However, conditioning electrical stimulation within the typical frequency band of C-fibers during inflammation (2 Hz) (13) did not change synaptic strength in any of the spino-PB neurons tested ( $108 \pm 19\%$  of control,  $n = 7$ ) (Fig. 2C). LFS, however, did modify synaptic strength in spinal lamina I neurons with a projection to the PAG. In all spino-PAG neurons tested, LFS induced a robust LTP of monosynaptic C-fiber-evoked EPSCs [to  $262 \pm 30\%$  of the control value at 30 min after stimulation ( $n = 18$ ) and to  $346 \pm 33\%$  at 60 min ( $n = 8$ )] (Fig. 2D). In all seven lamina I neurons with a projection to the PAG, conditioning stimulation at high frequency was ineffective ( $98 \pm 10\%$ ,  $n = 7$ ) (Fig. 2B). Monosynaptic, A-fiber-evoked

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EPSCs were not affected by conditioning LFS at C-fiber strength ( $113 \pm 5\%$  of the control value in four spino-PAG neurons tested).

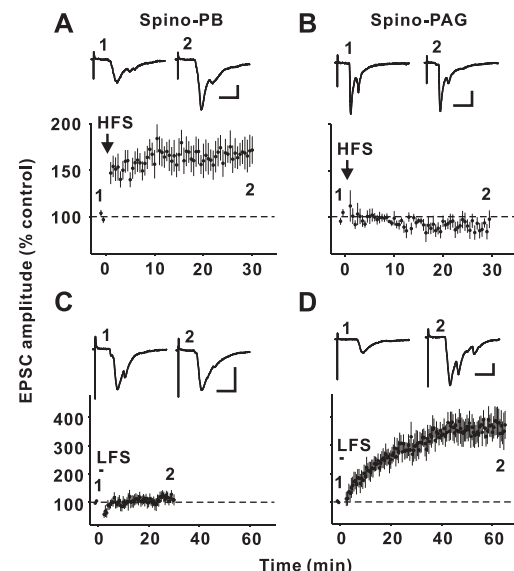


**Fig. 1.** Properties of lamina I projection neurons that express NK1Rs. Retrograde, fluorescent marker Dil (30 to 50 nL, 2.5%) was injected into either the PAG (A) or the PB (B) of 18- to 24-day-old rats. Shown are representative injection sites of two different animals on coronal sections. Black spots represent tissue damage due to the injections and red areas distribution of the dye. The distance from bregma is given in mm below each section; bar: 3 mm. LPB: lateral parabrachial area. (C) Recording sites of two lamina I neurons in lumbar spinal cord of two rats with a projection to the PAG (filled circle) or the PB (open circle) on a representative sketch of spinal dorsal horn (right-hand site; bar: 500  $\mu$ m). Both neurons responded with a transient inward current to bath application of substance P (horizontal bar: 2  $\mu$ M;  $V_{\text{hold}} = -60$  mV, left-hand site). Calibration bars: 30 s/20 pA. (D) Internalization of NK1Rs in spinal lamina I neurons following HFS (three bursts of 100 Hz, each given for 1 s at 10-s intervals) or LFS (2 Hz, 2 min) of sciatic nerve at C-fiber strength in intact, adult rats. Confocal images of representative transverse sections through the ipsilateral lumbar enlargement are shown. NK1R immunoreactivity is shown in green. Bars: 20  $\mu$ m.

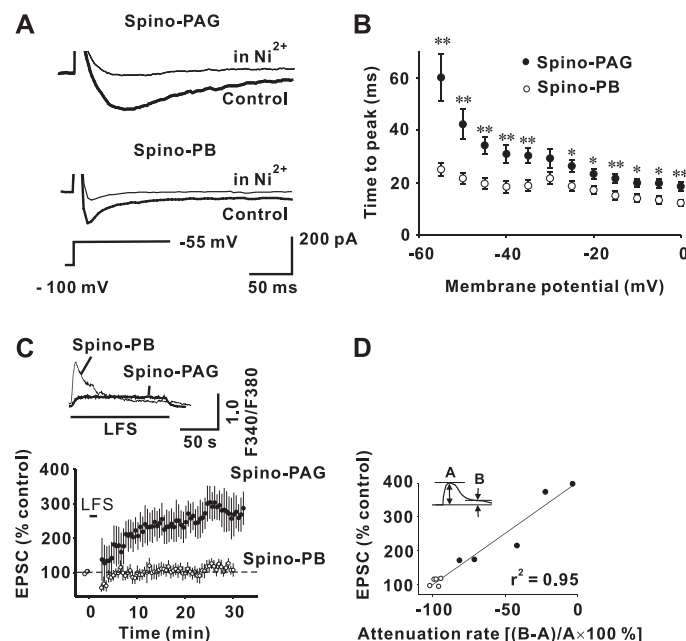
We next explored whether signal transduction pathways that are known to lead to hyperalgesia in vivo are also relevant for the induction of spinal LTP in vitro. In behaving animals, inflammatory hyperalgesia is prevented by spinal blockade of *N*-methyl-D-aspartate receptors (NMDARs), NK1Rs, or low-threshold, T-type voltage-gated calcium channels (VGCCs) (14, 15). Here, in-

duction of LTP by conditioning LFS also required activation of NK1Rs, NMDAR channels, and T-type VGCCs (fig. S1, A to C). This synergistically triggers a rise in postsynaptic cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and activation of phospholipase C (PLC), protein kinase C (PKC), and calcium-calmodulin-dependent protein kinase II (CaMKII), which

**Fig. 2.** Contrasting forms of LTP are expressed in distinct groups of spinal lamina I projection neurons. (A and B) Time courses of mean amplitudes ( $\pm$  SEM) of C-fiber-evoked EPSCs in lamina I neurons with a projection to the PB ( $n = 8$ ) or the PAG ( $n = 7$ ). Conditioning HFS induced LTP in all spino-PB neurons tested but was ineffective in spino-PAG neurons. (C and D) Conditioning LFS induced LTP in all 18 spino-PAG neurons tested but was ineffective in 7 spino-PB neurons.  $V_{\text{hold}} \approx -50$  mV; current-clamp mode was used during conditioning stimulation in all groups. Above the graphs are shown original, representative EPSCs recorded just before and 30 or 60 min after HFS or LFS, respectively. Calibration bars: 20 ms/200 pA.



**Fig. 3.** LFS-induced LTP in spino-PAG neurons requires sustained influx of  $\text{Ca}^{2+}$  into the cell. (A) Representative current traces evoked in a spino-PAG or a spino-PB neuron in response to a voltage step (bottom trace) and under control conditions (thick lines). Downward deflection (inward current) was sensitive to bath application of 100  $\mu$ M  $\text{Ni}^{2+}$  and had slower kinetics in spino-PAG than in spino-PB neurons (thin lines). (B) Activation kinetics of  $\text{Ca}^{2+}$  currents as determined from voltage-current curves. The mean time to peak ( $\pm$ SEM) of activation kinetics are plotted against test membrane potential and reveal slower activation kinetics for spino-PAG neurons ( $n = 15$ , filled circles) as compared to spino-PB neurons ( $n = 15$ , open circles). \* $P < 0.05$ , \*\* $P < 0.01$  as compared to spino-PB neurons. (C) Upper traces indicate  $\text{Ca}^{2+}$  signals of one spino-PAG neuron and one spino-PB neuron during LFS. The ratio of the intensities of fluorescence measured at 340 nm and 380 nm (F340/F380) is plotted against time. Graphs below show mean time courses of EPSC amplitudes (in percent of control values) in spino-PAG neurons or spino-PB neurons before and after LFS at time zero ( $n = 5$  in each group). In this group of neurons,  $\text{Ca}^{2+}$  imaging was performed in parallel with EPSC recordings. (D) A linear correlation exists between the change of EPSC amplitude (in percent of controls) and the attenuation rate. The attenuation rate of the  $\text{Ca}^{2+}$  signal varies for spino-PAG neurons but clusters around  $-100\%$  for spino-PB neurons. Open circles, spino-PB neurons; filled circles, spino-PAG neurons.



are all necessary for the full development of inflammatory hyperalgesia. Block of a  $\text{Ca}^{2+}$  rise in lamina I spino-PAG neurons (fig. S1D) or bath application of blockers of PLC, PKC, or CaMKII all abolished induction of LTP by LFS (fig. S1, E to G). Blockade of inositol-1,4,5-trisphosphate receptors ( $\text{IP}_3\text{Rs}$ ) converted LTP into LTD (fig. S1H).

A rise in  $[\text{Ca}^{2+}]_i$  may also activate nitric oxide synthase (NOS), which is essential for mediating inflammatory hyperalgesia in behaving animals (16). We therefore examined whether NO is essential for induction of LTP and which NO substrate is involved. Inhibition of NOS abolished LTP induction by LFS (fig. S2A). Soluble guanylyl cyclase (sGC), which is a major substrate for NO, was indispensable for LTP induction (fig. S2B). Immunohistochemically, we have identified sGC, but not NOS, in spino-PAG lamina I neurons (17), suggesting that NO has to cross the extracellular space to reach its target. Bath application of an NO scavenger effectively blocked LTP induction in spino-PAG neurons (fig. S2C).

The level and the time course of the  $[\text{Ca}^{2+}]_i$  rise in postsynaptic neurons determine the expression and polarity of synaptic plasticity (9, 18–20). We compared  $\text{Ca}^{2+}$  currents induced by depolarizing voltage steps from a holding potential ( $V_{\text{hold}}$ ) of  $-100$  mV in spino-PAG and spino-PB neurons in voltage-clamp experiments. Calcium currents in both groups had low activation thresholds ( $\approx -60$  mV from a  $V_{\text{hold}}$  of  $-90$  mV) and inactivation thresholds ( $\approx -75$  mV) and were abolished by  $\text{Ni}^{2+}$  (Fig. 3A), suggesting the contribution of T-type

VGCCs. The time to peak of activation kinetics was longer in spino-PAG neurons than in spino-PB neurons (Fig. 3B). We next used  $\text{Ca}^{2+}$  imaging to evaluate activity-dependent  $\text{Ca}^{2+}$  gradients. LFS applied to dorsal root induced a slower rise and fall in  $[\text{Ca}^{2+}]_i$  in spino-PAG neurons as compared to spino-PB neurons (Fig. 3C). The attenuation rate of  $[\text{Ca}^{2+}]_i$  was inversely correlated with the magnitude of LTP (Fig. 3D).

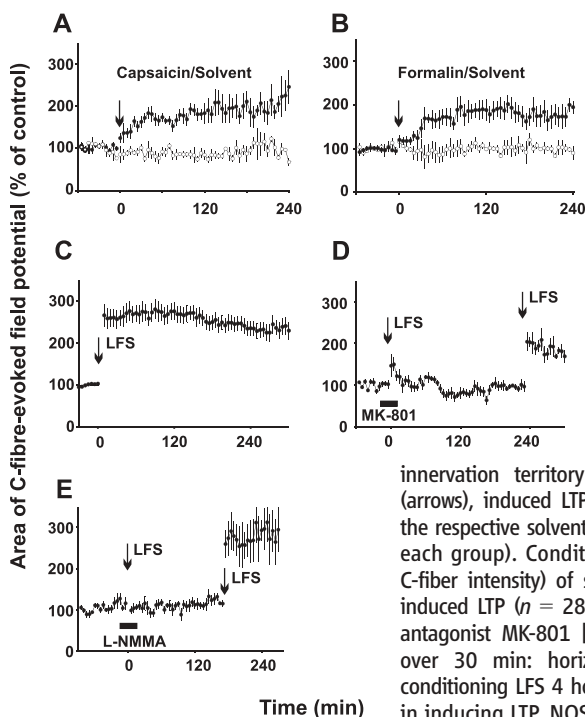
Presynaptic activity at low frequencies consistently fails to induce LTP unless the rise in postsynaptic  $[\text{Ca}^{2+}]_i$  is facilitated by removal of the voltage-dependent  $\text{Mg}^{2+}$  block of the NMDAR channel (9, 18–21). However, the induction of inflammatory hyperalgesia in vivo is triggered by irregular, asynchronous, low-frequency discharges in primary afferent C-fibers impinging on dorsal horn neurons, which are subject to considerable pre- and postsynaptic inhibition (14, 22). Thus, one might question whether a natural afferent barrage during inflammation or trauma can induce a sufficiently strong rise in  $[\text{Ca}^{2+}]_i$  in spinal neurons to trigger LTP in vivo. To address this key question, we monitored  $\text{Ca}^{2+}$  gradients in lamina I neurons during LFS or inflammation in vivo.

In 25- to 29-day-old intact rats, two-photon laser-scanning microscopy was used to quantify neuronal  $\text{Ca}^{2+}$  gradients in response to sensory stimulation (10). LFS of the sciatic nerve at C-fiber but not at A-fiber intensity induced a strong and sustained rise in  $\text{Ca}^{2+}$  concentration in all 27 lamina I neurons tested, including 7 neurons with an identified projection to the PAG (fig. S3, A and B). We next

investigated whether natural, low-frequency irregular and nonsynchronous discharges in a subset of nociceptive C-fibers can also raise  $\text{Ca}^{2+}$  concentrations in spinal lamina I neurons in vivo. Subcutaneous injection of capsaicin activates transient receptor potential vanilloid 1 receptor ion channels in a subset of nociceptive C-fiber afferents, leading to intense burning pain for a few minutes, followed by hyperalgesia for hours (7). Hyperalgesia evoked by capsaicin is commonly used to study the central mechanisms of pain amplification (7, 23). Here, capsaicin induced a strong rise in  $[\text{Ca}^{2+}]_i$  in 15 lamina I neurons tested (fig. S3C).

We then asked if conditioning stimuli that trigger sustained  $\text{Ca}^{2+}$  gradients in nociceptive lamina I neurons also amplify synaptic strength in vivo. We recorded C-fiber-evoked field potentials in the superficial spinal dorsal horn of adult, deeply anesthetized rats, with spinal cords intact (10). Capsaicin induced a slowly developing LTP to  $173 \pm 9\%$  of the control value ( $n = 5$ ) 60 min after injection (Fig. 4A). Subcutaneous injection of diluted formalin is a model of chemically induced inflammation and leads to long-lasting, low-frequency discharges in C-fibers and biphasic pain behavior in animals. Here, formalin injections induced a slow-onset LTP (to  $172 \pm 16\%$  of the control value at 60 min,  $n = 6$ ) (Fig. 4B). To exclude the possibility that ongoing activity in C-fibers contributed to the enhanced responses in spinal cord, we cooled the afferent nerve by a Peltier-element distal to the stimulation electrode 60 min after formalin injection. The nerve block did not affect maintenance of LTP in any of the three animals tested. Electrical LFS of sciatic nerve at C-fiber but not at A-fiber intensity ( $101 \pm 1\%$  of control,  $n = 3$ ) also induced a robust LTP in vivo (to  $274 \pm 21\%$  of the control value at 120 min and to  $228 \pm 20\%$  at 300 min,  $n = 28$ ) (Fig. 4C), which lasted up to 10 hours. In line with the in vitro results, LFS-induced LTP in vivo also required coactivation of NMDARs and NOS (Fig. 4, D and E). A-fiber-evoked field potentials were not potentiated by LFS at C-fiber strength in any of the eight animals tested.

We have identified a synaptic pain amplifier in the spinal cord that is turned on in mature animals by natural, asynchronous and irregular, low-rate discharge patterns in nociceptive C-fibers at synapses with spino-PAG neurons, a distinct subgroup of lamina I projection neurons (24–26). In contrast to other, rare forms of low frequency-induced LTP in the central nervous system, the synaptic plasticity described in this work can be induced under physiological conditions and in the presence of tonic pre- and postsynaptic inhibition. Our results suggest that during low-level presynaptic activity, multiple sources of  $\text{Ca}^{2+}$  are recruited simultaneously by activation of NMDARs, VGCCs, and NK1R and mobilization of  $\text{Ca}^{2+}$  from intracellular stores to achieve a sufficient rise of  $\text{Ca}^{2+}$  in the



**Fig. 4.** LTP can be induced by natural, low-frequency afferent barrage evoked by inflammation of peripheral tissue in vivo. Mean time courses of C-fiber-evoked field potentials recorded extracellularly in superficial spinal dorsal horn in response to electrical stimulation of left sciatic nerve of deeply anesthetized adult rats with spinal cords and afferent nerves intact. Subcutaneous injections of transient receptor potential vanilloid 1 channel agonist capsaicin (1%, 100  $\mu\text{l}$ ,  $n = 5$ ) (A) or formalin (5%, 100  $\mu\text{l}$ ,  $n = 6$ ) (B) into the glabrous skin at the ipsilateral hind paw, within the

innervation territory of the sciatic nerve at time zero (arrows), induced LTP (closed circles), whereas injections of the respective solvents (open circles) had no effect ( $n = 3$  in each group). Conditioning electrical LFS (2 Hz, 2 min at C-fiber intensity) of sciatic nerve at time zero (arrow) also induced LTP ( $n = 28$ ) (C), which was prevented by NMDAR antagonist MK-801 [3 mg  $\text{kg}^{-1}$ , intravenous (iv) infusion over 30 min: horizontal bar,  $n = 5$ ] (D). A second conditioning LFS 4 hours later (arrow) was partially effective in inducing LTP. NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) (100 mg  $\text{kg}^{-1}$  hour $^{-1}$ , iv infusion: horizontal bar,  $n = 5$ ) (E) also blocked LTP induction. This block was fully reversible, as shown by a second LFS 3 hours later (arrow).



postsynaptic neuron. This then leads to activation of calcium-dependent protein kinases and NOS, which causes amplification of pain-related information at the first synapse in pain pathways. Blockade of IP<sub>3</sub>Rs unmasked LTD of synaptic strength in C-fibers induced by LFS. This suggests that LFS may simultaneously induce synaptic plasticity of opposite polarity involving divergent signal transduction pathways. Hyperalgesia in human (27) and in animal studies (7, 28, 29) and the synaptic pain amplifier described in this work share induction mechanisms, relevant neuron populations in spinal cord, pharmacological profile, and signal transduction pathways. This strongly suggests that LTP at the first synapse in pain pathways between nociceptive C-fibers and spinal lamina I projection neurons is a cellular key mechanism of inflammatory hyperalgesia and perhaps other forms of low-level afferent-induced hyperalgesia (30, 31).

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#### Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5780/1659/DC1

Materials and Methods

Figs. S1 to S3

References

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## Food-Caching Western Scrub-Jays Keep Track of Who Was Watching When

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Western scrub-jays (*Aphelocoma californica*) hide food caches for future consumption, steal others' caches, and engage in tactics to minimize the chance that their own caches will be stolen. We show that scrub-jays remember which individual watched them during particular caching events and alter their recaching behavior accordingly. We found no evidence to suggest that a storer's use of cache protection tactics is cued by the observer's behavior.

Social living is suggested to have selected for increased sociocognitive skills in animals (1, 2). Several species use information about the dominance rank (3) and social relationships (4) of conspecifics when forming alliances or competing for resources (5, 6). Corvids rely on observational spatial memory to steal others' caches (7–9), and they engage in a variety of behaviors to reduce cache theft (10–15). It may be advantageous to remember who was watching during specific caching events, because scrub-jays can only defend caches against subordinates (16). Scrub-jays

should therefore engage in cache protection when they are caching in view of dominant birds, but might refrain from doing so when watched by their partner, because they often defend their partner's caches and tolerate cache theft by their mate (16).

Nine birds (storsers) cached in two trays, one near and one far from an observer. Storsers cached in the presence of a dominant bird ("dominant"), a subordinate bird ("subordinate"), their partner ("partner"), or when an observer's view was obscured ("in private"). After 3 hours, storsers recovered their caches "in private" (17). Partnerships and dominance indices were determined before this experiment when the birds were aviary housed (16).

The total number of items cached did not differ significantly between conditions [Friedman's analysis of variance (ANOVA),

$\chi^2_3 = 2.5$ ,  $P = 0.45$ ]. Birds cached predominantly in the far tray in the "dominant" (sign test:  $S = 7/7$ ,  $P = 0.02$ ) and "subordinate" condition ( $S = 7/7$ ,  $P = 0.02$ ), but not in the "partner" condition ( $S = 5/7$ ,  $P = 0.13$ ) or "in private" ( $S = 3/7$ ,  $P > 0.05$ ) (table S1).

The proportion of items that were recached at recovery was greatest in the "dominant" condition (Fig. 1A). In the "dominant" and "subordinate" conditions, items were predominantly moved from the near tray. Surprisingly, items were also moved, but from both trays, after the "in private" and "partner" conditions (Fig. 1B). Irrespective of condition, items were always moved to out-of-tray sites around the home cage.

The pattern of caching and recaching suggests that scrub-jays engage in cache protection to combat the specific risk that nonpartners pose to their caches, even though observers did not have the opportunity to pilfer. Specifically, birds cached mainly in the far tray when observed by a nonpartner and recached the few items hidden in the near tray at recovery. It may be difficult for observers to see the location of caches in the far tray, thereby decreasing their ability to use observational spatial memory to facilitate cache pilferage. This strategy was not implemented when the storer's partner was present, perhaps because storsers prefer to spend time near to their partner and do not perceive them as a risk to cache safety.

There are three alternate explanations for the preference to cache in the far tray in the "dominant" and "subordinate" conditions.

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